Optimizing Equilibration Protocols through Ubiquinone Supplementation in Goat Frozen Semen Diluent

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ABSTRACT

The equilibration period is essential for sperm to adjust to the diluent, thereby preventing severe damage during cryopreservation. The elevation of lipid peroxidation during equilibration, indicating potential oxidative stress involvement, underscores the importance of optimizing equilibration protocols to minimize the detrimental effects of free radical production on sperm quality. This study aims to investigate the supplementation effect of adding ubiquinone to semen diluent on the membrane integrity, motility, and survivability of goat spermatozoa following an hour of equilibration. Ubiquinone supplementation was administered at varying doses: Group 2 (B) received 5 mg/dL, Group 3 (C) received 10 mg/dL, Group 4 (D) received 15 mg/dL, Group 5 (E) received 20 mg/dL, and Group 6 (F) received 25 mg/dL. Group A served as the negative control. Following one-way ANOVA examination of the research data, additional Duncan tests were conducted. The study results revealed that ubiquinone addition significantly ($p<0.05$) affected the percentage of motility, viability, and membrane integrity of Kacang goat spermatozoa after one hour of equilibration. According to statistical analysis, the optimal concentration of ubiquinone in egg yolk skim milk to sustain motility is 12.5 mg/dL. Conversely, the most effective concentration for preserving the viability and membrane integrity of Kacang goat spermatozoa following equilibration is 25 mg/dL. In conclusion, the addition of ubiquinone to the frozen semen diluent maintains the stability of Kacang goat spermatozoa quality after equilibration.

Key words: *spermatozoa, equilibration, semen diluter, motility, viability, membrane integrity*

Introduction

Equilibration is the time required for spermatozoa to adjust to the diluent prior to freezing, preventing severe damage. This is why sperm equilibration plays a crucial role in assisted reproductive technologies for enhancing fertilization success and sperm function (Almeida et al, 2016). During sperm equilibration, the sperm cells are allowed to acclimate to the new environment, which helps optimize their function and increases the chances of successful fertilization. Furthermore, studies have shown that the duration of equilibration time greatly influences sperm motility and post-thaw survival rates (Málková et al., 2023). By allowing sufficient time for equilibration, sperm membranes can undergo repairs and protective mechanisms can be activated, resulting in reduced damage during the freezing-thaw. The equilibration period during the freezing of semen varies depending on individuals, the type of semen, diluent used, and the method of spermatozoa freezing (Bintara et al., 2021).

The mechanism by which equilibration impacts sperm quality is currently unknown. The first loss in sperm quality happens during the equilibration phase, which is amplified throughout the spermatozoa freezing process, which involves several significant temperature decreases (Priyanto et al., 2015). Nonetheless, balancing is thought to play a crucial role in preventing sperm membrane damage by protecting membrane components like cholesterol and mitochondrial membranes. It also helps prepare the sperm plasma membrane for low temperatures with an aim to maintain viability during cryopreservation (Vozaf et al., 2021).

Spermatozoa lose motility during the equilibration phase of the frozen semen production process because of the production of lactic acid from metabolic waste, which can lower pH levels and make the environment hazardous to spermatozoa (Fiqih et al., 2021). Prolonged equilibration periods may lead to sperm viability being significantly reduced due to damage to the spermatozoa plasma membrane. In addition, it may have an impact on the permeability of cell walls, which could result in sperm losing their motility. Throughout the equilibration process, variations in the amounts of internal and external fluid cause changes in the osmotic pressure of cells. This may lead to damage to cell membranes and the rupture of the lipoprotein envelope, perhaps resulting in aberrant spermatozoa.

Moreover, the elevation of lipid peroxidation during equilibration, indicating a potential involvement of oxidative stress, emphasizes the significance of optimizing equilibration protocols to minimize the detrimental effects of free radical production on sperm quality (Málková et al., 2023). Understanding the impact of equilibration time on free radical production and the antioxidant capacity of sperm is pivotal in developing strategies to mitigate oxidative stress and preserve sperm function during freezing and thawing processes. Sperm membrane is also vulnerable during equilibration due to the exposure to cold temperatures and the potential generation of superoxide, which can lead to peroxidation and damage to the phospholipid membrane. This makes it highly

dependent on the dilution content and integrity of the spermatozoa membrane during the equilibration process (Muhammad et al., 2020).

Optimizing equilibration protocols is paramount during the generation of frozen semen due to the urgent need to safeguard sperm viability and quality. During this critical period, sperm can adapt to the diluent environment, effectively preventing severe damage during the cryopreservation process (Benko et al., 2022). Failure to optimize equilibration protocols can lead to increased lipid peroxidation, indicating heightened oxidative stress, which significantly compromises sperm quality (Peris-Frau et al., 2020). Thus, the urgency lies in minimizing the detrimental effects of free radical production on sperm viability and functionality by refining equilibration procedures.

The role of nutrient content in the extender used during equilibration cannot be overstated (Muhammad et al., 2020). The availability of essential nutrients in the extender is vital for providing the necessary support for sperm viability and overall functionality during equilibration. This aspect underscores the importance of carefully formulating extenders to ensure the optimal environment for sperm acclimatization and preservation.

According to a number of studies (Appiah et al., 2020; Sharideh et al., 2019; Yousefian et al., 2018), supplementing the diluent can preserve the integrity of sperm cell membranes while also boosting motility, viability, and plasma membrane integrity. According to research published in 2016 by Saeed et al., the best dose for boosting motility, viability, and membrane integrity in post-equilibration buffalo and cow spermatozoa is antioxidant supplementation added to the diluent. This also reduces the number of abnormalities and protects against acrosome damage.

Like vitamins, ubiquinone is a fat-soluble molecule that is produced by mitochondria. Ubiquinone is also known as ubiquinone because it has a quinone group in its structure and is found in many kinds of cells, including plant and animal cells. When ubiquinone is intentionally added to the process of repairing cell damage caused by oxidative stress, it outperforms other antioxidants in this regard. Furthermore, ubiquinone has potent antioxidant qualities that allow it to

defeat cellular antioxidants in the defense against ROS (El-Sherbiny et al., 2022). Therefore, the purpose of this study is to ascertain how adding the antioxidant ubiquinone affects the membrane integrity, motility, and survivability of goat spermatozoa following equilibration.

Methods

The study has ethical permission and was conducted in August-November of 2023 at the Teaching Farm of the Faculty of Veterinary Medicine, Airlangga University, provided the samples of goat semen using artificial vagina, processing frozen semen, and analyzing the motility, viability, and membrane integrity of goat spermatozoa following equilibration using CASA software.

Fresh semen from a single Kacang goat, approximately two years old, was utilized as the sample. The goat's motility of fresh semen reached a minimum of 70%, indicating high fertility. The collected semen goes through several steps, such as dilution, cooling, and pre-freezing. Following that, at the Teaching Farm, Faculty of Veterinary Medicine, Airlangga University, CASA software is used to examine the motility and viability of spermatozoa.

Tools and materials

An artificial vagina, a cement storage container, a refrigerator, aluminum foil, analytical scales, beakers, magnetic stirrers, cover glass, hemocytometer, pH indicator paper, incubator, glassware, glass stick, water bath, heater, filter paper, filling, sealing, cool-top, straw rack, container freezing, sterile equipment, and a Windows XP computer with CASA software are among the tools used in this research. Vaginal gel, fresh goat bean semen, penicillin 100,000 IU, streptomycin 1 mg, egg yolk, fructose, glycerol 12%, glucose 2%, NaCl 0,9%, eosin-negrosine dye, ubiquinone (Nutrilab, USA), and Aquades were the components used in this study.

Frozen Semen Preparation

Egg yolk skim milk was used to generate the diluter in this study, which was then split into diluter A and B. In diluter A, ubiquinone supplementation was combined under the circumstances as follows: The groups with the following

supplementation doses: Group 2 (B) received a dose of 5 mg/dL; Group 3 (C) received a dose of 10 mg/dL; Group 4 (D) received a dose of 15 mg/dL; Group 5 (E) received a dose of 20 mg/dL; and Group 6 (F) received a dose of 25 mg/dL. Group A was the negative control group.

Examination of Spermatozoa Quality Post-Equilibration

According to Tethool et al. (2022), spermatozoa motility strongly related to membrane integrity, intracellular metabolic activity, and cell survival. Observing individual spermatozoa's motility requires utilizing a cover glass and a microscope with a 100x magnification. Computerized Assisted Semen Analysis (CASA) software was used to automatically track spermatozoa motility. Based on Ratnawati and Isnaini (2017), the evaluation of spermatozoa motility with the use of CASA produced more precise, impartial, consistent, and reproducible results.

Because spermatozoa viability and semen quality are interrelated, spermatozoa viability also serves as a determining indicator of semen quality. Spermatozoa membrane structure can be inferred from an examination of spermatozoa viability. The strength of the spermatozoa plasma membrane determines motility, which is associated with viability (Prastika et al., 2018). After equilibration, a 2% eosin-negrosin dye solution was added and dripped onto a sterile glass item to conduct a viability analysis. In order to determine the percentage of spermatozoa viability in Kacang goats, the viability of spermatozoa was directly calculated using a light microscope with a 400x magnification. This was combined with CASA to provide data in the form of percent (%).

Evaluation of membrane stability refers to the assessment of the spermatozoa membrane's integrity, which is crucial for regulating the spermatozoa's motility, viability, and transport system (Suryaningsih et al., 2020). The Hypo Osmotic Swelling Test (HOST) is used to examine the membrane integrity of spermatozoa. The total integrity of spermatozoa is then calculated using a light microscope with a 400x magnification that has been integrated with CASA software. The results are then obtained in the form of percent (%).

Stastical Analysis

The data analyzed is a comparison of all treatment groups based on the percentage of motility, viability and membrane integrity of the Kacang goat spermatozoa. The statistical analysis of the data involved conducting a homogeneity test followed by a One-way ANOVA using SPSS software version 29, then proceed with further tests using Duncan's multiple range test.

Result

Fresh Semen Evaluation

The volume, odor, color, pH, consistency and other macroscopic characteristics are examined to determine the suitability of fresh semen. In the meantime, supporting equipment like microscopes, glass objects, spectrophotometers and micropipettes are utilized for the microscopic evaluation of fresh semen. Table 1 displays the quality of fresh semen obtained based on the examination results in this study. The data analysis report compares the different treatment groups of Kacang goat spermatozoa based on their percentage of motility, viability, and sperm concentration.

Table 1. Results of the fresh semen quality inspection from Kacang goats

Parameters	Rresults
Volume (mL)	0,8 mL
Odor	normal
pH	7,1
Viscosity	Very viscous
Colour	normal
Mass Motility	++
Individual Motility (%)/ speed	82% / 3
Viability (%)	88%
Concentration (spermatozoa/ml)	2.200 million/mL

The analysis of the data revealed that the Kacang goat spermatozoa in this study exhibited high percentages of motility (82%) and viability (88%). The results of this research were higher than the percentage of fresh semen motility of Kacang goats according to (Kusumawati, 2022), namely 70%. The fresh semen viability average in this research was 88%, which exceeded the findings of Rokana et al., ranging from 83.38% to 88.94%. Based on the quality parameters of fresh semen tested and literature comparisons, the fresh semen from Kacang goats meets the feasibility criteria for making frozen semen.

Results of Observation of Kacang Goat Spermatozoa Motility After Equilibration

The motility of individual spermatozoa is best characterized by progressive or forward movement. The spermatozoa often experience cold shock, leading to backward or circular motion. Old semen is characterized by oscillatory movements, such as swinging or rotating in place. If many spermatozoa experience necrospermia or stop moving, this indicates that the spermatozoa are dead and no longer viable. Additionally, it can be inferred from various research

studies that fresh semen quality is an important factor in determining viability for processing into liquid or frozen semen. The robust forward movement of sperm cells is a crucial parameter in assessing the sperm population (Susilawati, 2011).

The results of this study aimed to compare the percentage of progressive motility of post-equilibration peanut goat spermatozoa given egg yolk skim milk diluent and added with ubiquinone at different concentrations. It is important to note that Kacang goat sperm is susceptible to cold temperature stress, resulting in a significant decrease in progressive motility after storage at 5°C, further emphasizing the need for careful handling and processing techniques. Additionally, maintaining a concentration of spermatozoa as per established standards will be crucial for ensuring high-quality semen production. Fresh cement that had been added to several different concentration treatments was subjected to post-equilibration observations. The results of these observations can be seen in **Table 2**.

Table 2. Kacang Goat Spermatozoa Percentage of Motility Results Following One Hour of Equilibration

Groups (ubiquinone mg/dL)	N	Average motility (%) ± SD
A (0 mg/dL)	5	$63,6 \pm 1,37^{a}$
B (5 mg/dL)	5	$70,2 \pm 2,00^{ab}$
C (10 mg/dL)	5	$72,4 \pm 1,21^{b}$
D (15 mg/dL)	5	$71,4 \pm 2,16^{b}$
E (20 mg/dL)	5	$69,6 \pm 1,26^{ab}$
F (25 mg/dL)	5	$69,3 \pm 1,17^{ab}$

Note: a, b, ab different superscript letters in the results show significant differences ($P<0,05$)

Based on the findings from assessing the movement of individual sperm cells, SPSS software was utilized to conduct an analysis for normality and homogeneity testing ($p>0,05$). It was followed by a one-way analysis of variance which indicated a highly significant difference ($p<0,05$) in the sperm motility percentage in Kacang goats after one hour equilibration. Based on the analysis of

the results, it can be concluded that adding ubiquinone at different concentrations to the egg yolk skim milk diluent had a significant effect on the motility of Kacang goat spermatozoa after equilibration.

The results of the Duncan test, as in **Table 2**, show that the A group had the lowest post-equilibration spermatozoa motility average value, namely 63,6% and was very significantly different (P < 0,05) from the C treatment group (72,4%) and D (71,4%) but less significantly different in B, E and F. As for each group given the dose, the lowest percentage of spermatozoa motility was in groups A (70,2%), E (69,6%) and F (69,3%) and was less significantly different (P<0,05) from the treatment group B and C. The highest mean percentage of spermatozoa motility was in the C treatment group (72,4%), followed by the D group (71,4%), which was significantly different from the control group (P < 0,05).

Result of the Viability (%) of Kacang Goat Spermatozoa After 1 hour of Equilibration

Spermatozoa membrane structure can be inferred from an examination of spermatozoa viability. The strength of the spermatozoa plasma membrane determines motility, which correlates with viability (Prastika et al., 2018). The eosin-nigrosin staining method was employed to evaluate the viability of Kacang goat spermatozoa after equilibration (Sun et al., 2022). The viability of Kacang goat spermatozoa after equilibration was assessed using the eosin-nigrosin staining method (Hiltpold et al., 2022). The desirable viability of spermatozoa is demonstrated when adding eosin-negrosin staining to the spermatozoa; live spermatozoa are identified by their transparent appearance as they do not absorb the dye. The non-living sperm cells will take up the eosin-negrosin stain, showing harm to the cell membrane and affecting its functionality. As a result, the dye permeates into the cell and stays within it, resulting in red coloring of the sperm cells, particularly at their heads (Susilowati et al., 2023).

Table 3. Kacang Goat Spermatozoa Percentage of Viability Results Following One Hour of Equilibration

Groups (ubiquinone mg/dL)	N	Average viability (%) ± SD

A (0 mg/dL)	5	75,4 ± 1,14[a]
B (5 mg/dL)	5	80,8 ± 3,19[b]
C (10 mg/dL)	5	80,6 ± 1,67[b]
D (15 mg/dL)	5	81,6 ± 1,78[b]
E (20 mg/dL)	5	81,2 ± 1,64[b]
F (25 mg/dL)	5	80,2 ± 1,30[b]

Note: a, b, ab different superscript letters in the results show significant differences (P<0,05)

Using SPSS software, the study findings from assessing spermatozoa viability were examined to perform homogeneity and normality test analysis (p>0,05). Subsequently, implementing One-way ANOVA on the addition of ubiquinone into the egg yolk skim milk diluent shown a highly significant (p<0,05) variation in the viability percentage of Kacang goat spermatozoa following an hour equilibration. **Table 3** shows that, compared to Group B, Group C, Group D, Group E, and Group F, the Negative Control group (A) had the lowest post-equilibration spermatozoa viability mean value (75,4%) and was significantly (P<0,05) different. With a significant difference from the control group (P<0,05), the P3 treatment group had the greatest average percentage of spermatozoa motility (81,6%), followed by the A group (80,8%), B group (80,6%), E group (81,2%), and F group (80,2%).

Discussion

Sperm motility decreases significantly due to cold shock during the equilibration process (Koelima et al., 2022). According to (Setyawan et al., 2019), During the equilibration process, spermatozoa generate energy through the breakdown of ATP within the mitochondrial envelope. This is facilitated by specific enzymes that stimulate the release of stored energy from phosphate bonds. Extended cold storage leads to membrane damage and prompts an enzymatic restoration of lost ATP, ultimately leading to reduced motility in spermatozoa. Damage to the plasma membrane can also disrupt sperm metabolism, resulting in a loss of sperm motility (Prayogo et al., 2022).

Based on the statistical analysis above, it has been found that adding ubiquinone to semen diluter positively influenced the percentage of individual spermatozoa motility after equilibration compared to the negative group (A). This is in agreement with previous findings and supports the utilization of ubiquinone to enhance sperm motility has been explored by El-Sherbiny et al., 2022, revealing that the addition of ubiquinone can play a crucial role in sustaining spermatozoa motility in goats. Apart from that, the addition of ubiquinone can also maintain the quality of individual motility after equilibration in several animals, including buffalo (Doidar et al., 2018), chickens (Masoudi et al., 2019), and cows (Saeed et al., 2016). The research findings suggest that adding ubiquinone to semen diluter has a positive impact on the individual spermatozoa motility after equilibration.

Research conducted by Saeed et al. in 2016 revealed that the addition of ubiquinone supplementation at a dose of 30 µM to frozen semen diluent significantly improved the individual spermatozoa motility after equilibration in both buffalo and cows. These findings highlight the potential impact of ubiquinone on maintaining sperm motility, suggesting its relevance in reproductive technologies for these animals. Where it can maintain individual motility up to 73%, the addition of ubiquinone to frozen semen diluent can increase metabolism and increase the synthesis of Adenosine Triphosphate (ATP), which is an essential energy-rich component used for spermatozoa motility.

Adding ubiquinone to the egg yolk skim milk diluent increased the percentage of spermatozoa viability after equilibration according to the statistical study presented above. This study validates other research showing that ubiquinone supplementation can preserve spermatozoa viability in goats (El-Sherbiny et al., 2022). Additionally, after equilibration, ubiquinone can preserve the viability of spermatozoa in a variety of animals, such as wild boar (74%; Pindaru et al., 2014), buffalo (59.53%; Doidar et al., 2018), chicken (68%; Masoudi et al., 2019), cattle (73.90%; Saeed et al., 2016), and horses up to 83.80% (Yousefian et al., 2018).

According to (Saeed et al., 2016), adding ubiquinone at an appropriate concentration of 30µM to frozen semen diluent is the most effective dose in sustaining the percentage of post-equilibration spermatozoa viability in cattle. It can keep spermatozoa viable for up to 76% of the time. The antioxidant ubiquinone in frozen sperm diluent can suppress the development of hydroperoxides, protecting the plasma membrane from oxidation during the equilibration process. By inhibiting lipid oxidation in the sperm cell membrane, the integrity of the plasma membrane is preserved, allowing sperm cells to withstand freezing (Olivieri, 2019).

Thus, the findings of this study are consistent with previous findings that the addition of ubiquinone to frozen sperm diluent has an effect on preserving spermatozoa viability parameters after an hour equilibration with the most optimal additional dose, namely 25 mg/dL, with an average percentage of spermatozoa viability reaching 81,6%.

Result of the Sperm's Membrane Integrity (%) of Kacang Goat Spermatozoa After 1 hour of Equilibration

The spermatozoa membrane's condition maintains control over water transport to prevent external fluids from entering the cell, ensuring membrane integrity (Lestari et al., 2014). Membrane integrity evaluation is an examination of the integrity of the spermatozoa membrane, which controls the motility, viability, and transport system of spermatozoa (Suryaningsih et al., 2020). Spermatozoa with excellent membrane integrity have curved tails or swelling of the spermatozoa **Figure 1.**, whereas spermatozoa with low membrane integrity have straight tails or do not bulge (Susilawati, 2011). **Table 4** display the result of the Sperm's Membrane Integrity (%) after an hour of equilibration.

Table 4. Kacang Goat Spermatozoa Sperm's Membrane Integrity Results Following One Hour of Equilibration

Groups (ubiquinone mg/dL)	N	Average Membrane Integrity (%) ± SD
A (0 mg/dL)	5	67,6 ± 1,81[a]
B (5 mg/dL)	5	70,4 ± 1,14[ab]

C (10 mg/dL)	5	70,2 ± 1,09^{ab}
D (15 mg/dL)	5	72,6 ± 1,67^{b}
E (20 mg/dL)	5	71,2 ± 1,78^{b}
F (25 mg/dL)	5	71 ± 1,58^{b}

Note: a, b, ab different superscript letters in the results show significant differences (P<0,05)

Table 4 illustrates that the group A exhibited the lowest post-equilibration spermatozoa membrane integrity mean value of 67.6%, showing very significant difference (P < 0,05) compared to group D (72.6%), group E at 71.2%, and group F at 71%, but a less significant difference in Group B and Group C. Based on the data, the P3 treatment group had the highest average percentage of spermatozoa membrane integrity at 72.6%, followed by P4 at 71.2% and P5 at 71%. These values were significantly different from those of the control group (P < 0,05).

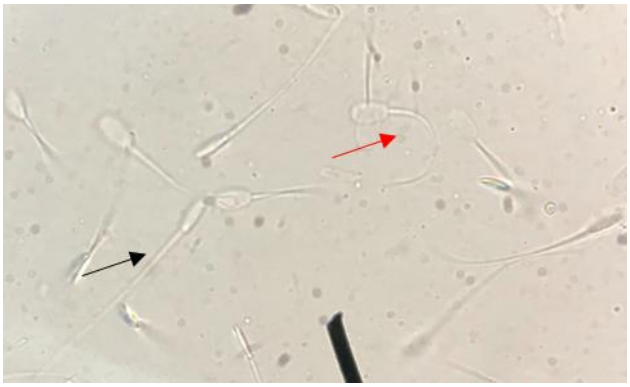

Figure 1. Sperm's Membrane Integrity using hypo-osmotic swelling Test (1000x). Notes: red arrow indicates normal membran integrity; black arrow indicates abnormal membrane integrity

During the equilibration process, the integrity of the spermatozoa membrane is crucial for maintaining water transport and preventing damage from cold shock. During the equilibration process, sperm cells can be vulnerable to cold shock, potentially compromising the integrity of their plasma membrane. During this procedure, nitric oxide has the capability to stimulate superoxide generated by sperm while utilizing oxygen. An excess of superoxide results in peroxidation within the phospholipid membrane of sperm cells, leading to functional impairment. Research reported by Susilowati et al, (2014) shows that the

equilibration process for 1 hour can initiate up to 32% damage to the spermatozoa membrane. The type of diluent and the duration of equilibration time are leading factors in damaging the spermatozoa membrane (Yendraliza et al., 2019).

One mechanism by which ubiquinone reduces free radicals during cryopreservation is through its antioxidant properties. Ubiquinone, in its reduced form (ubiquinol), acts as a cellular antioxidant and scavenges free radicals and reactive oxygen species, preventing them from causing oxidative damage to cellular components (Thelin et al., 1992). Additionally, ubiquinol can also regenerate other antioxidants, such as α-tocopherol (vitamin E), further enhancing the cellular antioxidant defense system during cryopreservation. Ubiquinone can also diminish superoxide radicals produced in the electron transport chain of mitochondria (Jové et al., 2013).

This helps to prevent the formation of harmful reactive oxygen species and protects cellular membranes from lipid peroxidation, which can lead to cell membrane damage. Therefore, the presence of ubiquinone in its reduced form helps to maintain the antioxidant capacity and integrity of cells during certain condition, reducing the potential for oxidative damage and promoting successful preservation of biological materials. MitoQ antioxidant, which contains the redox forms of ubiquinone (reduced mitoquinol and reduced mitoquinone), can be used as a supplement during cryopreservation to enhance the antioxidant defense system and further reduce free radical damage (Oliver & Reddy, 2019).

Conclusion

In conclusion, the addition of ubiquinone to the frozen sperm diluent has been found to effectively preserve spermatozoa viability, motility and membrane integrity parameters after equilibration. Recent research has consistently supported the conclusion regarding the beneficial effects of ubiquinone supplementation in frozen sperm diluent. Studies have demonstrated that ubiquinone supplementation effectively preserves sperm viability, motility, and membrane integrity parameters following equilibration. These findings align with previous research, which has reported positive impacts of ubiquinone on sperm quality and viability. Moreover, current research underscores the intricate

mechanisms through which equilibration influences sperm quality, emphasizing its significance in fertility preservation and the success of assisted reproductive technologies (ART).

Furthermore, ongoing research efforts continue to shed light on the complexities of equilibration and its effects on sperm function and integrity. This ongoing exploration underscores the importance of refining equilibration protocols and deepening our understanding of its implications for sperm viability and post-thaw qualities. By advancing our knowledge in this area, we can enhance techniques and technologies crucial for successful fertilization and the preservation of fertility, both in human and animal reproductive contexts. Therefore, the quest for optimizing equilibration protocols remains essential for further advancements in ART and the continuous improvement of reproductive outcomes.

References

Cheema, R. S., Bansal, A. K., & Bilaspuri, G. S. (2009). Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility. Oxidative medicine and cellular longevity, 2(3), 152–159. https://doi.org/10.4161/oxim.2.3.8804

Bintara, S., Astuti, A., Panjono, P., & Kusumastuti, T. A. (2021). Effect of different pre-freezing time on quality of frozen fat-tailed ram semen. Buletin Peternakan, 45(3), 155. https://doi.org/10.21059/buletinpeternak.v45i3.65426

Almeida, F C., Silva, S V., Souza, H M D., Gomes, W A., Filho, J A., Wicke, A A., Batista, A M., & Guerra, M M P. (2016, June 2). Effects of glycerol, equilibration time and antioxidants on post-thaw functional integrity of bovine spermatozoa directly obtained from epididymis. https://doi.org/10.1111/and.12623

Amal, A S., Arifiantini, R I., Setiadi, M A., & Said, S. (2019, June 20). Characteristics of the post-thawed Balinese bull semen extended in three different extenders and equilibration times. https://doi.org/10.14710/jitaa.44.2.135-145

Priyanto, L., Arifiantini, R. I., & Yusuf, T. L. (2015). Detection of Spermatozoa DNA Damage in Fresh and Frozen Bovine Semen Using Toluidine Blue Staining. Jurnal Veteriner, 16(1), 48–55. http://repository.ipb.ac.id/handle/123456789/71904

Vozaf, J., Makarevich, A V., Baláži, A., Vašíček, J., Svoradová, A., Olexiková, L., & Chrenek, P. (2021, January 1). Cryopreservation of ram semen: Manual versus programmable freezing and different lengths of equilibration. https://scite.ai/reports/10.1111/asj.13670

Fiqih, A. F., Soeharsono, S., Yunita, M. N., Dhamayanti, Y., & Yudhana, A. (2021). Toxic Effect of Robusta Coffee Extract (Coffea canephora) on Bali Cattle Spermatozoa After Equilibration. Jurnal Medik Veteriner, 4(2), 189. https://doi.org/10.20473/jmv.vol4.iss2.2021.189-192

Muhammad, M., Eriani, K., Fithri, A., Rusdi, A., & Saputra, H. (2020, January 1). Potential of Flamboyant Flower (Delonix regia(Boj. ex Hook) Raf) Extract to Maintain Post-Dilution Quality of Aceh Cattle (Bos taurus indicus) Spermatozoa. https://doi.org/10.1051/e3sconf/202015101044

Benko, F., Ďuračka, M., Lukáč, N., & Tvrdá, E. (2022, October 12). CRYOCAPACITATION AND ITS ASSOCIATION WITH OXIDATIVE FEATURES IN CRYOPRESERVED BOVINE SPERMATOZOA. Journal of microbiology, biotechnology and food sciences, e9268-e9268. https://doi.org/10.55251/jmbfs.9268

Peris-Frau, P., Soler, A J., Iniesta-Cuerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D A., Fernández-Santos, M., García-Álvarez, O., Maroto-Morales, A., Montoro, V., & Garde, J. (2020, April 16). Sperm Cryodamage in Ruminants: Understanding the Molecular Changes Induced by the Cryopreservation Process to Optimize Sperm Quality. International Journal of Molecular Sciences, 21(8), 2781-2781. https://doi.org/10.3390/ijms21082781

Sharaf, A E., Khalil, W A., Khalifa, E I., Nassan, M A., Swelum, A A., & El-Harairy, M. (2022, October 27). The Supplementation of Bee Bread Methanolic Extract to Egg Yolk or Soybean Lecithin Extenders Can Improve the Quality of Cryopreserved Ram Semen. https://doi.org/10.3390/cells11213403

Vozaf, J., Makarevich, A V., Baláži, A., Vašíček, J., Svoradová, A., Olexiková, L., & Chrenek, P. (2021, January 1). Cryopreservation of ram semen: Manual versus programmable freezing and different lengths of equilibration. https://scite.ai/reports/10.1111/asj.13670

Sun, L., He, M., Xu, J., Wu, C., Zhang, S., Zhang, D., Dai, J., & Gao, J. (2022, November 17). Does Antioxidant Mitoquinone (MitoQ) Ameliorate Oxidative Stress in Frozen–Thawed Rooster Sperm?. https://scite.ai/reports/10.3390/ani12223181

Hiltpold, M., Janett, F., Mapel, X M., Kadri, N K., Fang, Z., Schwarzenbacher, H., Seefried, F R., Spengeler, M., Witschi, U., & Pausch, H. (2022, March 7). A 1-bp deletion in bovine QRICH2 causes low sperm count and immotile sperm with multiple morphological abnormalities. https://scite.ai/reports/10.1186/s12711-022-00710-0

Longobardi, V., Zullo, G., Cotticelli, A., Salzano, A., Albero, G., Navas, L., Rufrano, D., Claps, S., & Neglia, G. (2020). Crocin Improves the Quality of Cryopreserved Goat Semen in Different Breeds. Animals : an open access journal from MDPI, 10(6), 1101. https://doi.org/10.3390/ani10061101

Jové, M., Ayala, V., Ramírez-Núñez, O., Naudí, A., Cabré, R., Spickett, C M., Portero-Otin, M., & Pamplona, R. (2013, April 30). Specific Lipidome

Signatures in Central Nervous System from Methionine-Restricted Mice. https://scite.ai/reports/10.1021/pr400064a

Oliver, D., & Reddy, P H. (2019, April 1). Small molecules as therapeutic drugs for Alzheimer's disease. https://scite.ai/reports/10.1016/j.mcn.2019.03.001

Thelin, A., Schedin, S., & Dallner, G. (1992, November 23). Half-life of ubiquinone-9 in rat tissues. https://scite.ai/reports/10.1016/0014-5793(92)81425-l

Málková, A., Savvulidi, F., Ptáček, M., Machová, K., Janošíková, M., Nagy, S., … & Stádník, L. (2023). Glycerol-free equilibration with the addition of glycerol shortly before the freezing procedure: a perspective strategy for cryopreservation of wallachian ram sperm. Animals, 13(7), 1200. https://doi.org/10.3390/ani13071200

El-Sherbiny, H. R., Abdelnaby, E. A., El-Shahat, K. H., Salem, N. Y., Ramadan, E. S., Yehia, S. G., & Fathi, M. (2022). Coenzyme Q10 Supplementation enhances testicular volume and hemodynamics, reproductive hormones, sperm quality, and seminal antioxidant capacity in goat bucks under summer hot humid conditions. Veterinary Research Communications, 46(4), 1245–1257. https://doi.org/10.1007/s11259-022-09991-8

Appiah, M. O., Asante-Badu, B., Zhao, J., Liu, H., Wang, J., & Lu, W. (2020). Possible Protective Mechanisms of Coenzyme Q10 Action on Spermatozoa During Cryopreservation or Cooled-Stored Condition. Journal CryoLetters, 41(5), 246–256

Sharideh, H., Zhandi, M., Zenioaldini, S., Zaghari, M., & Sadeghi, M. (2019). The effect of coenzyme Q10 on rooster semen preservation in cooling condition. Theriogenology, 129, 103–109. https://doi.org/10.1016/j.theriogenology.2019.02.028

Yousefian, I., Emamverdi, M., Karamzadeh-Dehaghani, A., Sabzian-Melei, R., Zhandi, M., & Zare-Shahneh, A. (2018). Attenuation of cryopreservation-induced oxidative stress by antioxidant: Impact of Coenzyme Q10 on the quality of post-thawed buck spermatozoa. Cryobiology, 81, 88–93. https://doi.org/10.1016/j.cryobiol.2018.02.005

Yendraliza, Y., Yuliana, E., Rodiallah, M., & Zumarni, Z. (2019). Kualitas Semen Kerbau pada Waktu Ekuilibrasi dan Inkubasi yang Berbeda dalam Larutan Hipoosmotic Swelling TEST. Jurnal Agripet, 19(1), 22–30. https://doi.org/10.17969/agripet.v19i1.13191

Susilowati, S., Sardjito, T, and I. N. Triana. (2014). Addition of Cow Seminal Plasma on Quality Goat Spermatozoa in The Freezing Process with Different Equilibration Times. Ovozoa (Jurnal Reproduksi Hewan), 3(2). https://doi.org/10.20473/ovz.v8i2.2019.110-115

Suryaningsih, H., Sabdoningrum, E. K., Susilowati, S., Sardjito, T., Wurlina, W., & Damayanti, T. (2020). Penambahan L – Arginin Dalam Pengencer Tris Kuning Telur Setelah Ekuilibrasi 2 Jam Terhadap Motilitas Dan Membran Plasma Utuh Spermatozoa Domba Sapudi. Ovozoa : Journal of Animal Reproduction, 7(2), 86. https://doi.org/10.20473/ovz.v7i2.2018.86-90

Doidar, Y. A., L-Nagar, H. A., & Elrefy, A. (2018). Cryopreservation and Quality Assessment of Buffalo Bull (*Bubalus bubalis*) Semen Using New Moringa

Extender and Antioxidant Co-q10. Journal Animal and Poultry, 9(9), 375–381. https://dx.doi.org/10.21608/jappmu.2018.41144

Koelima, B. V. W., Belli, H. L. L., Kihe, J. N., & Nalley, W. M. (2022). Effect of equilibration time on frozen sement quality of the duroc pig in tris diluents modification with the addition of cryoprotectants. Jurnal Nukleus Peternakan, 9(1), 92–100. https://doi.org/10.35508/nukleus.v9i1.5490

Masoudi, R., Sharafi, M., & Pourazadi, L. (2019). Improvement of rooster semen quality using coenzyme Q10 during cooling storage in the Lake extender. Cryobiology, 88, 87–91. https://doi.org/10.1016/j.cryobiol.2019.03.003

Saeed, A., El-Nagar, H., Wafa, W., & Hussein, Y. (2016). Effect of Coenzyme Q10 as an Antioxidant Added to Semen Extender During Cryopreservation of Buffalo and Cattle Semen. Journal of Animal and Poultry Production, 7(11), 403–408. https://doi.org/10.21608/jappmu.2016.48748

Prayogo, D., Susilowati, S., Prastiya, R. A., Safitri, E., & Agustono, B. (2022). Difference Time Effect of Equilibration Before Freezing on The Quality of Spermatozoa Sapera Goats using Egg Yellow Tris. Jurnal Medik Veteriner, 5(2), 188–195. https://doi.org/10.20473/jmv.vol5.iss2.2022.188-195

Lestari, W. A. D., Susilowati, S., & Warsito, S. H. (2014). The Effect Of Various Thawing Time On Frozen Semen of Fat Tailed Sheep for Viability, Motility and Intact Plasma Membrane of Spermatozoa. Ovozoa, 3(2)

Prastika, Z., Susilowati, S., Agustono, B., Safitri, E., Fikri, F., & Prastiya, R. A. (2018). Motility and Viability of Rambon Cow Spermatozoa in Kemiren Village, Banyuwangi. Jurnal Medik Veteriner, 1(2), 38. https://doi.org/10.20473/jmv.vol1.iss2.2018.38-42

Ratnawati, D., & Isnaini, N. (2017). Utilization of CASA in Observing Spermatozoa Motility of Madurese Cow Liquid Semen in Different Diluents. Jurnal Ilmu-Ilmu Peternakan, 27(1), 80–95. https://doi.org/10.21776/ub.jiip.2017.027.01.07

Tethool, A. N., Ciptadi, G., Wahjuningsih, S., & Susilawati, T. (2022). Karakteristik dan Jenis Pengencer Semen Sapi Bali: Suatu Review: Bali Cattle Semen Characteristics and Diluent Types: A Review. Jurnal Ilmu Peternakan dan Veteriner Tropis (Journal of Tropical Animal and Veterinary Science), 12(1). https://doi.org/10.46549/jipvet.v12i1.214